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In re New U.S. Continuation Appln. of:	
Pascal DESMAZEAU et al.	Parent Group Art Unit: 1653
Application No.: 10/790,260	Parent Examiner: D. Lukton
Filed: March 2, 2004	
For: STREPTOGRAMIN DERIVATIVES, PREPARATION METHOD AND COMPOSITIONS CONTAINING SAME	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. 1.132

I, Dr. Nadine BERTHAUD, declare and state that:

1. I am a French citizen, residing at 20 rue de Marne, 94140 Alfortville, France.

2. I have been awarded the degree of Doctor of Veterinary Medicine from Ecole Nationale Vétérinaire de Maisons-Alfort, and have a diploma from the Institut Pasteur for a course entitled Systematic Microbiology.

3. I have been employed by Aventis Pharma S.A., formerly Rhone-Poulenc Rorer, S.A., ("Aventis") since 1977 and until 2003 I was the Head of Antibacterial Microbiology in the Infectious Disease Group at Aventis. During this employment at

Aventis, I have been engaged in applied research and development regarding potential antibacterial compounds and I was responsible for the evaluation of the *in vitro* and *in vivo* activity of new antibacterial agents.

4. Given my education and experience, particularly in the area of antibacterial compounds, I consider myself qualified to provide the following testimony based on the below-described experiments related to U.S. Patent Application No. 10/790,260 ("the '260 application"), conducted by me or under my direct supervision.

5. Given my education and experience, I also consider myself qualifed to provide the following testimony concerning the common names of Streptogramin A compounds.

6. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the '260 application or any patent issuing thereon.

I. Testing

Streptogramin compounds according to general formula (I) in U.S. Patent Application No. 10/790,260, were tested for *in vitro* and *in vivo* activity against the bacteria *S.aureus (in vitro), S.aureus Schiclia* (*in vitro*) and *S.aureus IP8203 (in vivo).* As described further below, the testing included measurement of the activity of compounds according to general formula (I) tested (1) *in vitro* against exemplary

bacteria (*S.aureus, S.aureus Schiclia*) to determine a minimum effective concentration, both individually and in combination with pristinamycin IIB ("PIIB"), and (2) *in vivo* against an exemplary bacteria (*S.aureus IP8203*) to determine a 50% curative dose in combination with each of dalfopristin and PIIB, via subcutaneous and oral routes, respectively.

The testing procedures were as follows:

***In vitro* bacteriostatic activity**

The bacteriostatic activity of the compounds of general formula (I) of the '260 application was determined according to the U.S. standards (Antimicrobial Susceptibility Testing: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. 1992 National Committee for Clinical Laboratory Standards, M7-A2, Villanova, PA.).

Two-fold dilutions of the 1280 mg/l antibacterial stock solution tested were added to molten Mueller-Hinton agar supplemented with 25 mg/l Mg++ and 50 mg/l CA++ (1 part of antibacterial solution for 9 parts of liquid agar), then poured into plates. A multipoint inoculator was used to apply spots of about 10^4 colony forming units (cfu) of each strain tested onto agar. After inoculation, plates were incubated 18 hours at 37°C.

The minimum inhibitory concentration ("MIC") was defined as the lowest concentration (µg/ml) that completely inhibited the growth of bacteria.

For the combination treatments (*i.e.,* test compound in combination with PIIB), a 30/70 weight/weight ratio of test compound to PIIB was used.

***In vivo* antibacterial activity in the model of *staphylococcus aureus* mouse septicemia**

Mice (6 to 8 per group) were inoculated intraperitoneally with 0.5 ml of the bacterial strain cultured under shaking in Brain Heart Infusion at 37°C and diluted in 7.5% porcine mucin so as to obtain about 10^6 cfu/ml. Under these conditions, infected untreated controls die in 24 to 48 hours.

Each compound tested was administered by the subcutaneous (s.c.) or the oral (p.o.) routes twice on the day of inoculation, the first dose being given 1 hour after infection and the second dose 6 hours after infection.

The test compositions contained a compound according to general formula (I) with PIIB or dalfopristin, as indicated in the following table, in a 30/70 weight/weight ratio of the formula (I) compound to P IIB or dalfopristin.

The vehicle was an aqueous solution or a suspension in 0.9% NaCl aqueous solution added with 0.1% polysorbate 80 (Prolabo). The administered volume was 1 ml/mouse per treatment.

Three to 6 doses of up to 150 mg/kg were used.

The Curative Dose 50 ("DC_{50}") (mg/kg), calculated 7 days post infection, was defined as the dose which protected 50% of the infected treated mice from death when all the infected untreated controls died.

II. Results

The results of the testing procedures above as applied to compounds according general formula (I) of the '260 application are presented in the following table. For reference, results for PIIB and dalfopristin alone are also provided. In the table, the

example numbers refer to the compounds of the corresponding examples in the '260 application.

Example No.	*In Vitro S.aureus 209P* MIC (μg/ml)		*In Vitro S.aureus Schiclia* MIC (μg/ml)		*In Vivo S.aureus IP8203* DC_{50} (mg/kg)	
	Compound Alone	With PIIB	Compound Alone	With PIIB	S.C. with dalfopristin	P.O. with PIIB
1	8	0.5	>128	2	120	32
2	2	0.25	>128	1	28	32
3	2	0.25	>128	1	32	28
4	2	0.25	>128	1	32	32
5	4	0.25	128	1	30	30
6	16	1	>128	2	38	95
7	64	1	>128	4	75	36
8	8	1	>128	2	32	90
9	16	1	>128	2	5	100
10	64	4	>128	4	46	150
11	4	0.25	>128	0.5	90	100
12	2	0.5	>128	0.5	36	100
13	128	1	>128	2	85	100
14	2	0.25	>128	4	<5	42
15	4	0.5	>128	1	42	50
16	4	0.5	>128	1	42	32
17	4	0.25	>128	1	46	110
18	8	0.5	>128	1	36	34
19	8	0.5	>128	1	36	34
20	4	0.5	128	1	40	50
21	8	0.5	>128	1	90	75
22	128	2	>128	4	100	110
23	>128	1	>128	2	>150	100
24	8	1	>128	2	36	44
25	4	0.5	>128	1	32	26

Example No.	*In Vitro S.aureus 209P* MIC (µg/ml)		*In Vitro S.aureus Schiclia* MIC (µg/ml)		*In Vivo S.aureus IP8203* DC_{50} (mg/kg)	
	Compound Alone	With PIIB	Compound Alone	With PIIB	S.C. with dalfopristin	P.O. with PIIB
26	2	0.25	>128	1	40	32
27	32	1	>128	4	32	110
28	4	0.25	>128	1	32	40
29	4	0.5	>128	1	10	110
30	4	0.25	>128	1	32	15
31	16	1	>128	2	15	100
32	4	0.25	>128	0.5	120	44
33	4	0.25	>128	0.5	8	50
34	64	2	>128	2	85	110
35	8	1	>128	2	8	50
PIIB alone		4		4		> 300
dalfopristin alone					> 300	

III. Analysis

As shown by the results, *in vitro*, streptogramin compounds according to general formula (I) have proven active against *Staphylococcus aureus* 209P at concentrations of as low as 1 µg/ml, and in combination with pristinamycin IIB, have proven active at concentrations of 0.25 to 10 µg/ml. Additionally, *in vitro*, compounds according to general formula (I) in combination with PIIB have proven effective against *S. aureus Schiclia* at concentrations ranging from 0.5 to 4 µg/ml. In the combination treatments, the results show that, in nearly every instance, the activity of the combination is enhanced over either the streptogramin formula (I) compound or PIIB, when tested individually. For instance, against *S.aureus 209P*, the MIC activities of compound 1 and

PIIB, tested individually, were 8 and 4 µg/ml, respectively, while in combination the MIC activity was 0.5 µg/ml. Further, against *S.aureus Schiclia*, the MIC activities of compound 1 and PIIB, tested individually, were >128 and 4 µg/ml, respectively, while in combination the MIC activity was 2 µg/ml.

In vivo, streptogramin compounds according to general formula (I) have proven effective against *Staphylococcus aureus* IP 8203 test infections in mice in subcutaneous doses of 25 to 150mg/kg combined with dalfopristin, and with orally administered doses of 15 to 150mg/kg combined with pristinamycin IIB.* These results can be compared to an activity of > 300 mg/kg for dalfopristin and pristinamycin IIB, when each is tested individually in analogous *in vivo* tests. Thus, the combination treatments were more potent in their DC_{50} than both dalfopristin and pristinamycin IIB taken individually.

* For compound 23, the *in vivo* activity (DC_{50}) with dalfopristin was only determined to be greater than 150 mg/kg. Higher doses were not tested to more precisely determine the DC_{50}.

IV. Common names of Streptogramin A compounds

In my experience, streptogramin A compounds are often identified by common names, and in my opinion one skilled in the art would understand the reference to a streptogramin A compound based on its common name. In this regard, I am aware of several references that expressly refer to streptogramin A compounds by their common names. *See, e.g.*, J.C. Barrière et al., Current Pharmaceutical Design, 4, 155-180 (1998) (referring at pg. 156 to, *inter alia*, pristinamycin II_A, II_B, II_C, II_D, II_E, II_F, and II_G); V. Blanc et al., J. Bacteriology 177 (18), 5206-5214 (1995) (discussing streptogramin compounds including pristinamycin II_A and II_B).

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Cloning and Analysis of Structural Genes from *Streptomyces pristinaespiralis* Encoding Enzymes Involved in the Conversion of Pristinamycin II_B to Pristinamycin II_A (PII_A): PII_A Synthase and NADH:Riboflavin 5′-Phosphate Oxidoreductase

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In *Streptomyces pristinaespiralis*, two enzymes are necessary for conversion of pristinamycin II_B (PII_B) to pristinamycin II_A (PII_A), the major component of pristinamycin (D. Thibaut, N. Ratet, D. Bisch, D. Faucher, L. Debussche, and F. Blanche, J. Bacteriol. 177:5199–5205, 1995); these enzymes are PII_A synthase, a heterodimer composed of the SnaA and SnaB proteins, which catalyzes the oxidation of PII_B to PII_A, and the NADH:riboflavin 5′-phosphate oxidoreductase (hereafter called FMN reductase), the SnaC protein, which provides the reduced form of flavin mononucleotide for the reaction. By using oligonucleotide probes designed from limited peptide sequence information of the purified proteins, the corresponding genes were cloned from a genomic library of *S. pristinaespiralis*. SnaA and SnaB showed no significant similarity with proteins from databases, but SnaA and SnaB had similar protein domains. Disruption of the *snaA* gene in *S. pristinaespiralis* led to accumulation of PII_B. Complementation of a *S. pristinaespiralis* PII_A^- PII_B^+ mutant with the *snaA* and *snaB* genes, cloned in a low-copy-number plasmid, partially restored production of PII_A. The deduced amino acid sequence of the *snaC* gene showed no similarity to the sequences of other FMN reductases but was 39% identical with the product of the *actVB* gene of the actinorhodin cluster of *Streptomyces coelicolor* A(3)2, likely to be involved in the dimerization step of actinorhodin biosynthesis. Furthermore, an *S. coelicolor* A(3)2 mutant blocked in this step was successfully complemented by the *snaC* gene, restoring the production of actinorhodin.

Pristinamycin belongs to the family of streptogramin antibiotics, also called virginiamycin-like or mikamycin-like antibiotics. Streptogramins are a small and homogeneous group composed of related compounds such as pristinamycin, virginiamycin, mikamycin, and vernamycin (9, 10, 58). They are protein synthesis inhibitors (9, 10). The special feature of the family is that each member is a complex of two structurally different components exhibiting a synergistic antibacterial activity (2, 10). The two types of compounds are both macrocyclic lactone peptolides, but their structures are notably different. They belong to one of the two following distinct groups: the streptogramin A type (Sa) corresponding to polyunsaturated cyclic peptolides and the streptogramin B type (Sb) corresponding to branched cyclic hexadepsipeptides. The proportion of Sa and Sb in the complex varies from one antibiotic to another. Moreover, the major form of each component is accompanied by several structurally different minor forms.

Pristinamycin, produced by *Streptomyces pristinaespiralis*, consists of approximately 30% pristinamycins I (PI), the Sb type molecules, and 70% pristinamycins II (PII), the Sa type molecules. In industrial strains, PII is produced mainly in two forms, PII_A and PII_B, in a 80:20 ratio. The difference between PII_A and PII_B is the presence of a dehydroproline instead of a proline in the macrocycle (Fig. 1). Thibaut et al. (57) reported high levels of conversion of radiolabelled PII_B to PII_A both in vivo and in vitro with several strains of *Streptomyces* spp. that produce pristinamycins. The same type of observation was made with *Streptomyces virginiae*, the producer of virginiamycin, closely related to pristinamycin (49). These results indicated that PII_B is the biosynthetic precursor of PII_A, and so the oxidation of the proline residue into a dehydroproline residue appears to be the last step of PII_A biosynthesis.

Thibaut et al. (57) also showed that two enzymes are involved in the conversion of PII_B to PII_A (Fig. 1). Both were purified to homogeneity. The first, called PII_A synthase, is a heterodimer composed of two polypeptides, SnaA and SnaB, with M_rs of 50,000 and 35,000, respectively. It catalyzes the oxidation of the proline residue of PII_B in the presence of molecular oxygen and reduced flavin mononucleotide ($FMNH_2$). The second is an NADH:riboflavin 5′-phosphate oxidoreductase (hereafter called FMN reductase), SnaC, with an apparent M_r of 30,000 which provides the reduced FMN necessary for the oxidation of PII_B.

In this study, we describe the cloning, sequencing, and characterization of the structural genes for PII_A synthase (*snaA* and *snaB*) and FMN reductase (*snaC*) from *S. pristinaespiralis* and provide evidence for their functions. We believe that this is the first report of the cloning of genes involved in the synthesis of a streptogramin.

MATERIALS AND METHODS

Bacterial strains, phages, cosmids, and plasmids. The bacterial strains, phages, cosmids, and plasmids are listed in Table 1.

Media and bacteriological techniques. *Streptomyces* strains were maintained on HT agar medium (48) and grown in YEME medium (28) at 30°C. Liquid cultures for pristinamycin production were prepared by the method of Thibaut et al. (57), with an inoculum step of 44 h and a production step of 32 h. Extraction

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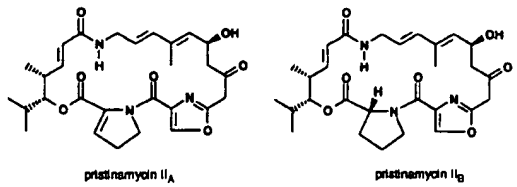
(A)



(B)

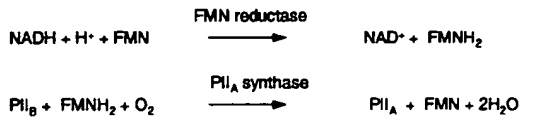


FIG. 1. (A) Structures of PII$_A$ and PII$_B$. (B) Reactions catalyzed by PII$_A$ synthase and FMN reductase.

and quantitation of the PII components were performed as described elsewhere (57).

Nosiheptide was used as an alternative to thiostrepton to select for the presence of the *tsr* gene, at a concentration of 400 μg/ml for solid media and of 2 μg/ml for liquid media. *Escherichia coli* strains were grown in LB medium at 37°C (44). Selection was made with 100 μg of ampicillin per ml in LB agar or liquid media.

DNA isolation and manipulation. Total DNA from *S. pristinaespiralis* SP92 was obtained by lysozyme treatment and phenol-chloroform extraction as described by Hopwood et al. (28). Plasmid DNA was purified by alkaline extraction procedures as described by Hopwood et al. (28) for *Streptomyces* species and by Maniatis et al. (44) for *E. coli*. Single-stranded DNA was extracted by the phenol-chloroform procedure (44) and dialyzed against water for 45 min prior to sequencing. Digestion with restriction endonucleases and ligation experiments were carried out by standard procedures (44) under conditions described by the manufacturer. DNA fragments were isolated from agarose gels with the Geneclean kit from Bio101 (La Jolla, Calif.).

Transformations. Competent *E. coli* cells were prepared and transformed by the method of Chung and Miller (8). For transformation, *S. pristinaespiralis* and *S. coelicolor* cells were grown in YEME medium supplemented with 0.25 and 0.5% glycine, respectively, at 30°C for 40 h. Protoplasts were prepared and transformed by the method of Hopwood et al. (28). Only unmethylated DNA, isolated from *E. coli* ET12567, was used for transformation of *S. coelicolor* (41).

DNA-DNA hybridization. Transfer of denatured DNA from agarose gels or colonies to Biodyne nylon membranes (Pall Corporation, Portsmouth, England) were performed by standard procedures (44). DNA fragments were labelled by random priming with [α-^{32}P]dCTP by using the random primer labelling kit (Amersham International, Little Chalfont, Buckinghamshire, England), as described by the supplier. Oligonucleotide probes were labelled with [γ-^{32}P]dATP with T4 polynucleotide kinase by the method of Maniatis et al. (44). Hybridization experiments were performed by the method of Maniatis et al. (44).

Oligonucleotide probes. As previously reported (57), the N-terminal sequences of SnaA, SnaB, and SnaC are TAPR(R/W)RITLAGIIDGPGG, TAPIL VATLDTRGPAATLGTIT, and TGADDPARPAVGPQSFRDAMAQLASPV, respectively. Internal sequences obtained by tryptic digestion (57) were identified as GADGFNIDFPYLPGSADDFV for SnaA, GL(-)DSFDDDAFVHDR for SnaB, and FAGGEFAAWDGTGVPYLPDAK and TGDPAKPPLLWYR for SnaC. Degenerate primers or oligonucleotide probes derived from part of the N-terminal or internal sequence of SnaA (IDFPYLPG), SnaB (FDDDAFVH), and SnaC (FRDAMAQLA, FAGGEFAAWDGTG, and DPAKPPLLWYR) were synthesized and are as follows: (degenerate positions shown in parentheses): A, 5′-ATCGA(C,T)TT(C,T)CC(C,G,A,T)TA(C,T)CT(C,G)CC(C,G)GG-3′; B, 5′-TTCGACGA(T,C)GA(T,C)GC(A,T,C,G)TTCGT(C,G)CA(T,C)GA C-3′; C1, 5′-TTCCG(C,G)GACGC(C,G)ATGGC(C,G)CAGCT(C,G)GC-3′; C2, 5′-TTCGC(C,G)GG(C,G)GG(C,G)GAGTTCGC(C,G)GC(C,G)TGGGA CGGCAC(C,G)GG-3′; and C3, 5′-GACCC(C,G)GC(C,G)AAGCC(C,G)CC (C,G)CT(G,C)CT(G,C)TGGTACCG-3′, respectively.

Preparation of antiserum. Rabbits were immunized by repeated subcutaneous inoculations of the two subunits of the purified PII$_A$ synthase. The protocol was based on three injections of 100 μg of proteins (in complete Freund adjuvant at days 0, 15, and 30) and one injection of the same dose (in incomplete Freund adjuvant at day 37). Blood was harvested 10 days after the last injection.

Preparation of cell extracts. Portions (5 ml) of *S. pristinaespiralis* cell suspensions were harvested after 16, 18, 20, or 22 h of culture in production medium (57). The washing buffer was phosphate-buffered saline (44) supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 5 mM ethylene glycol-bis(β-amino ethyl) tetraacetic acid (EGTA). The pellet was kept frozen at −20°C. Prior to sonication, cells were thawed and resuspended in 1.5 to 2 ml of the same buffer. Cells were disrupted with the Bioruptor type UEC-200 (Eurogentec, Seraing, Belgium) by the following procedure: four rounds of 5-min oscillating pulses (48 s on, 24 s off; power of 200 W). The obtained lysate was centrifuged for 15 min in an Eppendorf tube at 10,000 × *g*, and the resulting supernatant was referred to as cell extract. Protein concentration was determined by the method of Lowry et al. (39).

Assays of PII$_A$ synthase and FMN reductase activities. Enzymatic activities were assayed from cell extracts obtained with cells from 30 ml of fermentation broth, as described elsewhere (57).

Western blot (immunoblot) analysis. Proteins, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (36), were electroblotted onto nitrocellulose membranes [Cellulosenitrat(E); Schleicher and Schuell, Dassel, Germany) by using the Biometra Fastblot (Biometra Inc., Tampa, Fla.). Antigenic proteins were stained by using the Vectastain ABC Mouse IgG kit (Vector Laboratories, Biosys S.A., Compiègne, France) and anti-rabbit immunoglobulin G-horseradish peroxidase conjugate according to the procedures suggested by the manufacturer.

Construction of *S. pristinaespiralis* genomic library. A partial *Sau*3A digestion of *S. pristinaespiralis* genomic DNA was fractionated on a 20 to 40% sucrose gradient as described by Maniatis et al. (44). DNA fragments (35 to 45 kb) were ligated with pHC79 linearized with *Bam*HI. In vitro packaging with the Gigapack II Gold Packaging Extract (Stratagene, La Jolla, Calif.) was performed as described by the manufacturer, using HB101 or DH1 as the recipient strain. A total of 1,500 colonies for each transfection were selected on LB agar supplemented with ampicillin. Selected clones were individually grown in 200 μl of Hogness medium (19) in 96-well microplates and stored at −80°C.

DNA sequence analysis. A 4-kb *Sac*I-*Bam*HI fragment from pXL2045 containing the *snaA* and *snaB* genes was digested with different restriction enzymes (*Sac*I, *Not*I, *Nru*I, *Eco*RI, *Pst*I, and *Bam*HI). The resulting DNA fragments were subcloned in M13mp18 and M13mp19 vectors. The nucleotide sequence of the corresponding single-stranded DNA was determined by the dideoxy-chain termination method (51) with universal and synthetic oligonucleotides primers. Reactions were performed with dye-labelled dideoxy terminators from the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and analyzed on the Applied Biosystems model 370A DNA Sequencer (Applied Biosystems). In the case of the *snaC* gene, a 1.5-kb *Xho*I-*Pst*I fragment included in the 4-kb *Bam*HI-*Bam*HI fragment from pVRC509 was cloned in M13mp18 and M13mp19 and was partially sequenced as described previously with universal and synthetic primers.

Analysis of sequence data. Nucleic acid and amino acid sequences were analyzed by using CITI2 facilities (13). The nucleotide sequences were analyzed by the program of Staden and McLachlan (55), using codon preference to identify the coding sequences. A codon preference table was established with 19,673 codons from *Streptomyces* species, obtained from GenBank. Amino acid sequences were compared with Genbank, NBRF, and Swissprot databases by using either the FASTA (13) or Kanchisa (31) program. Multiple alignments were performed with the CLUSTAL multiple-alignment program of Higgins and Sharp (23).

Integrative transformation of *S. pristinaespiralis*. The *snaA* gene was disrupted by homologous recombination by an integration construction containing a fragment internal to the N-terminal part of the gene. A 800-bp *Pst*I-*Eco*RI fragment was subcloned from pXL2045 in the suicide vector pIJ15 to create pVRC505. The recombinant plasmid was used to transform *S. pristinaespiralis*, and recombinants were selected for the ability to grow on nosiheptide-containing plates. After 7 days, the resistant colonies were passed through one step of single-colony purification on HT medium containing nosiheptide.

Homologous expression of *snaA* and *snaB* in *S. pristinaespiralis*. Because the *snaA* gene started 31 bp after the *Bam*HI site, we isolated a 7.3-kb *Sac*I fragment from pIBV1, corresponding to an extra 3-kb fragment upstream of the *snaA* gene. This fragment was first subcloned in pUC1813 to give pVRC506. The 7.3-kb fragment was then isolated from pVRC506 by *Hin*dIII digestion and cloned in *Hin*dIII-linearized pIJ903. The recombinant plasmid, named pVRC507, contained *snaA* and *snaB* downstream of the *tet* promoter of pIJ903, albeit separated from each other by ORF401 oriented in the opposite direction from that of *snaA* and *snaB*.

Heterologous expression of *snaC* in *S. coelicolor*. The 1.5-kb *Xho*I-*Pst*I fragment containing the *snaC* gene and the 3′ end of the upstream open reading frame (ORF) was isolated from pVRC509 and cloned into pUC19 linearized by double digestion with *Sal*I and *Pst*I, giving pVRC518. A DNA fragment containing the *ermE** promoter from *Saccharopolyspora erythraea* (6) was purified from pVRC1116 after digestion with *Eco*RI and *Bam*HI and cloned into pVRC518 digested with *Eco*RI and *Bam*HI. The recombinant plasmid was named pVRC519. The *Eco*RI-*Hin*dIII fragment containing the *snaC* gene under control of the *ermE** promoter was purified and cloned in pIJ903 linearized by digestion with *Eco*RI and *Hin*dIII. The recombinant plasmid was named pVRC520. Transformation of *E. coli* ET12567 with pVRC520 allowed the preparation of unmethylated DNA necessary for transformation of *S. coelicolor*.

TABLE 1. Bacterial strains, phages, cosmids, and plasmids used

Strain, phage, cosmid, or plasmid	Relevant properties	Source or reference
Strains		
E. coli		
HB101	F⁻ *supE44 hsdS3*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	7
DH1	F⁻ *gyrA96 recA1 relA1 endA1 thi-1 hsdR17 supE44*	38
TG1	K-12 Δ(*lac-pro*) *supE thi hsd* ΔS5/F′ *traD36 proA*⁺*B*⁺ *lacI*^{q} *lacZ*ΔM15	20
DH5α	F⁻ *E44 ΔlacU169 φ80 lacZ*ΔM15 *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	22
ET12567	F⁻ *dam-13*::Tn*9 dcm-6 hsdM hsdR recF143 zjj-202*::Tn*10 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtl-1 glnV44*	42
S. pristinaespiralis		
SP92	Natural isolate of *S. pristinaespiralis* ATCC 25486	Rhône-Poulenc Rorer
SP119	PI^- PII_A^+ PII_B^+; mutant of *S. pristinaespiralis* SP92 obtained by chemical mutagenesis	Rhône-Poulenc Rorer
SP120	PI^- PII_A^- PII_B^+; mutant of *S. pristinaespiralis* SP119 obtained by chemical mutagenesis	
S. coelicolor A3(2)		
B135	*hisA1 uraA1 strA1* SCP1⁻ SCP2⁺ *actVB-235*	50
Phages		
M13mp18, M13mp19	Multicloning site vector	Boehringer
Cosmids		
pHC79	Cosmid; Amp^r	26
pIBV1	Cosmid containing the PII_A synthase genes; Amp^r	This work
pIBV3	Cosmid overlapping with pIBV1; Amp^r	This work
pIBV4	Cosmid containing the FMN reductase gene; Amp^r	This work
Plasmids		
pUC18, pUC19	Multicloning site vector; Amp^r	Biolabs
pUC1813	Multicloning site vector; Amp^r	33
pBKS⁻	Multicloning site vector; Amp^r	Stratagene
pIJ702	*Streptomyces* high-copy-number plasmid; *mel* Tsr^r	32
pIJ903	*E. coli* and *Streptomyces* shuttle vector (low-copy-number); Amp^r Tsr^r	40
pDH5	*Streptomyces* suicide vector; Amp^r Tsr^r	25
pXL2045	6-kb *Bam*HI-*Bam*HI insert from pIBV1 in pBKS⁻ containing *snaA* and *snaB*; Amp^r	This work
pVRC509	4-kb *Bam*HI-*Bam*HI insert from pIBV4 in pUC19 containing *snaC*; Amp^r	This work
pVRC505	800-bp *Pst*I-*Eco*RI insert from pXL2045 in pDH5; Amp^r Tsr^r	This work
pVRC506	7.3-kb *Sac*I-*Sac*I insert from pXL2045 in pUC1813; Amp^r	This work
pVRC507	*Streptomyces* expression vector containing the entire *snaA* and *snaB* genes in pIJ903; Amp^r Tsr^r	This work
pVRC1116	*ermE** promoter region cloned in pIC20H from pUC1070; Amp^r	12
pVRC518	1.5-kb *Xho*I-*Pst*I insert from pVRC509 in pUC19; Amp^r	This work
pVRC519	*ermE** promoter cloned upstream *snaC* in pVRC518; Amp^r	This work
pVRC520	*Streptomyces* expression vector of *snaC*, cloned in pIJ903; Amp^r Tsr^r	This work

Nucleotide sequence accession number. The nucleotide sequences from *S. pristinaespiralis* described in this paper have been submitted to GenBank under accession numbers U21215 for the region containing *snaA*, *snaB*, and ORF401 and U21216 for the region containing *snaC*.

RESULTS

Identification and cloning of the *snaA* and *snaB* genes. Oligonucleotide probes A and B were synthesized on the basis of internal amino acid sequences of the SnaA and SnaB proteins of the PII_A synthase, respectively. They were used to screen 3,000 colonies of the genomic library of *S. pristinaespiralis* SP92 by colony hybridization. Five clones hybridizing with either one or both probes were identified. Four of the recombinant cosmids contained a 6-kb *Bam*HI fragment hybridizing with both probes. One clone, named pIBV1, with a 33-kb insert, was studied further. The fifth clone, named pIBV3, with a 34-kb insert, did not contain the 6-kb *Bam*HI fragment described previously, but as pIBV1, a 2.5-kb *Eco*RI fragment hybridizing with probe A only. Restriction maps of these two cosmids were constructed (Fig. 2). They shared a 8-kb region containing the 2.5-kb *Eco*RI fragment. The 6-kb *Bam*HI fragment from pIBV1 was cloned in pBKS⁻ to give pXL2045 (Fig. 2). The nucleotide sequence of 3,573 bp from the 4-kb *Sac*I-*Bam*HI fragment from pXL2045 was determined as described in Materials and Methods. Analysis of the obtained nucleotide sequence revealed three ORFs (ORF1, ORF2, and ORF3) with a typical *Streptomyces* codon usage, ORF2 being on the strand opposite to that carrying ORF1 and ORF3 (Fig. 2). ORF1, ORF2, and ORF3 encoded polypeptides of 422, 401 or 402, and 277 amino acids, with M_rs of 46,500, 45,200, and 28,700, respectively (Fig. 3). Typical Shine-Dalgarno sequences (56) (GGAG, GGAG, and AGGA) were found upstream of ORF1, ORF2, and ORF3, respectively (Fig. 3), indicating that in the case of ORF2, the GTG is most probably the start codon. No significant inverted repeat was found between the intergenic regions or at the end of ORF3.

The N-terminal region of ORF1 was identical to the N-terminal amino acid sequence of the purified large subunit of

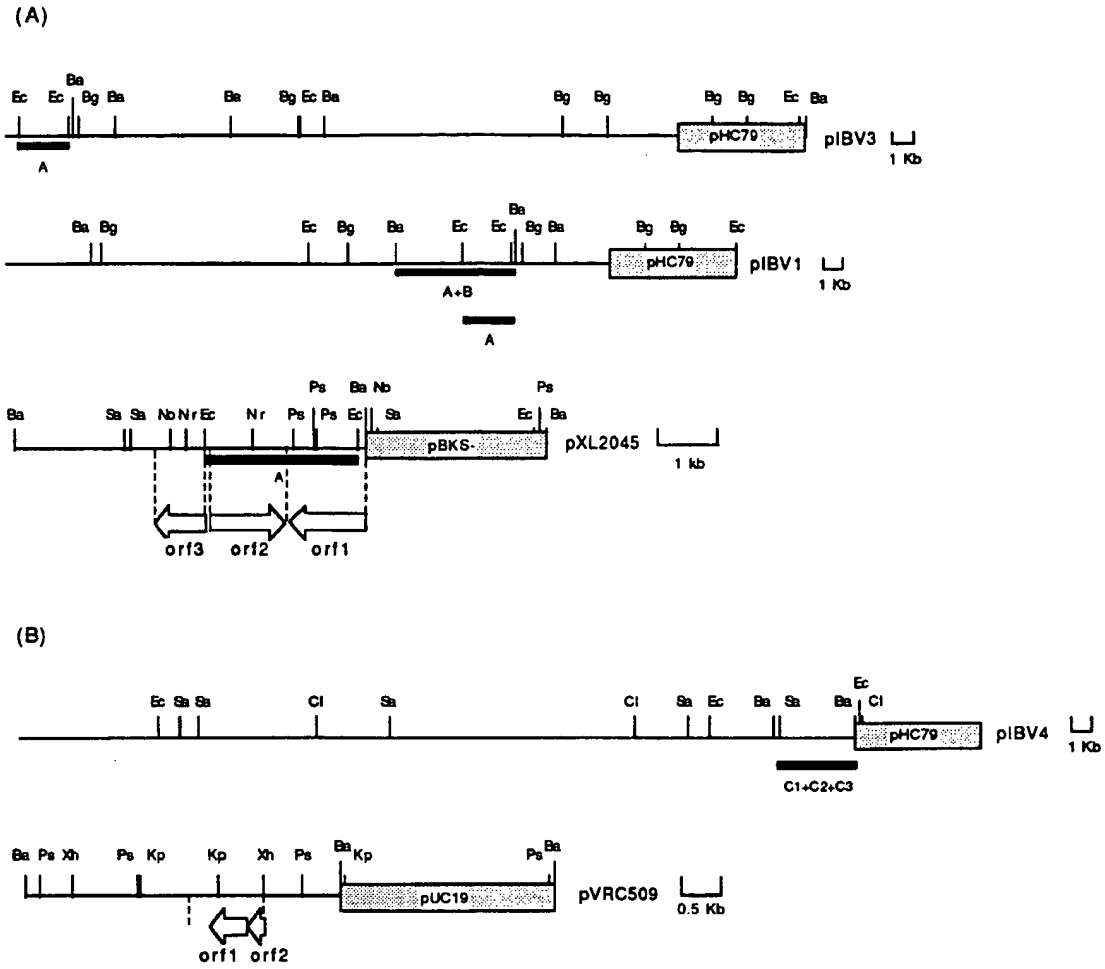


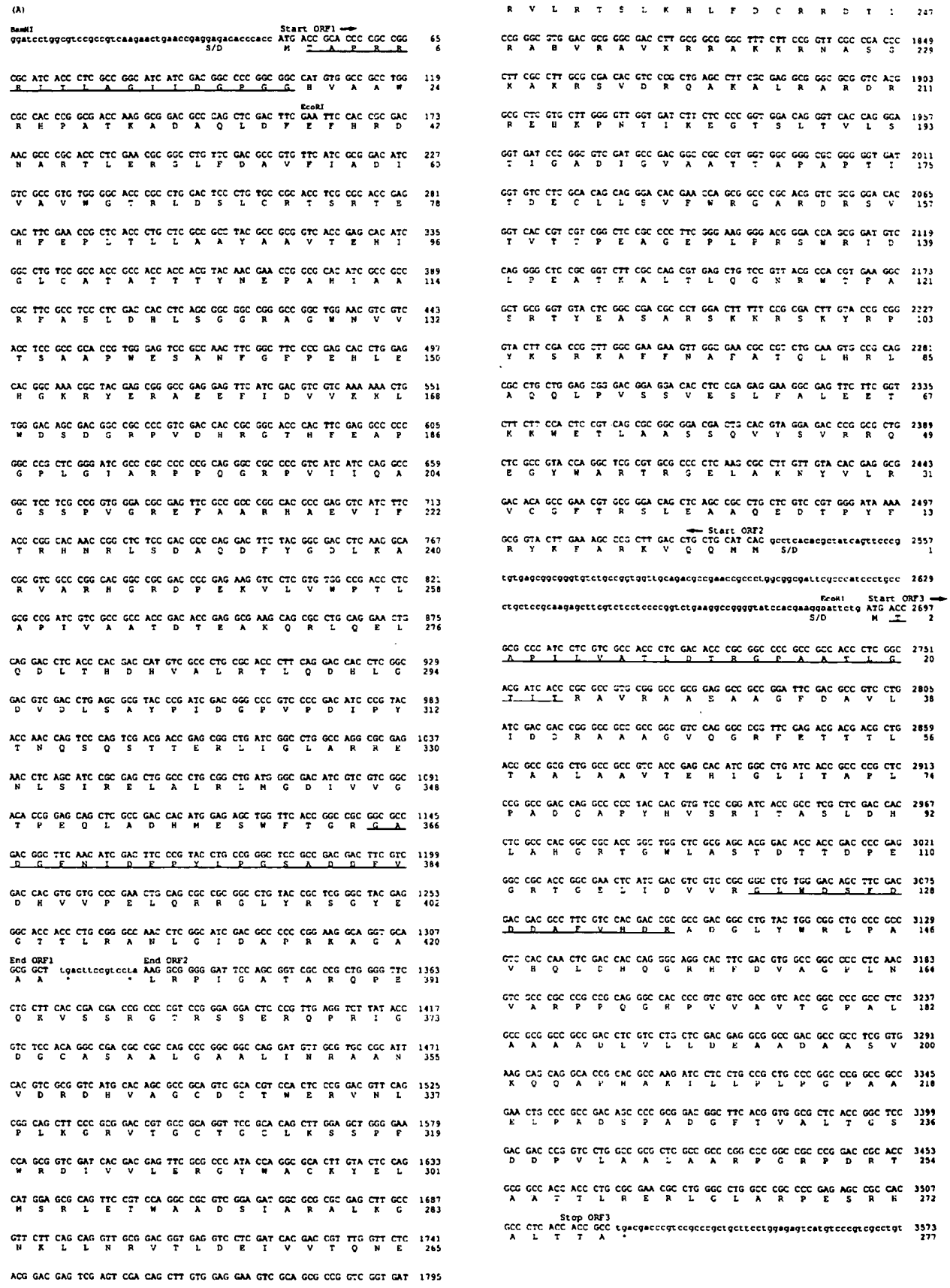
FIG. 2. (A) Restriction maps of cosmids pIBV1 and pIBV3 and the pXL2045 vector containing the 6-kb *Bam*HI fragment from pIBV3. (B) Restriction map of cosmid pIBV4 and the pVRC509 vector containing the 4-kb *Bam*HI fragment from pIBV4. Arrows correspond to the identified ORFs. The black boxes show the fragments hybridizing with probes described in the text. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; Cl, *Cla*I; Ec, *Eco*RI; Kp, *Kpn*I; No, *Not*I; Nr, *Nru*I; Ps, *Pst*I; Sa, *Sac*I; Xh, *Xho*I.

the PII$_A$ synthase, except that the amino-terminal methionine was missing (removal of the methionine residue has been proposed to occur when the penultimate amino acid is threonine [24]). The N-terminal region of the ORF3 product was identical with the N-terminal amino acid sequence of the purified small subunit of PII$_A$ synthase. Moreover, the internal amino acid sequences obtained from tryptic digestion of SnaA and SnaB were found in the polypeptides encoded by ORF1 (amino acids 365 to 384) and ORF3 (amino acids 122 to 136). A good correlation was observed between the calculated M_rs of the ORF1 and ORF3 products, respectively, 46,500 and 28,700, and the ones estimated from the purified subunits of PII$_A$ synthase, 50,000 and 35,000, respectively (57).

These results demonstrated that ORF1 and ORF3 corresponded to the large and small subunits of PII$_A$ synthase, and we named the corresponding genes *snaA* and *snaB*. They were separated by 1.4 kb containing ORF2. ORF2 was named ORF401 for the size of the corresponding polypeptide. The average G+C content of the sequenced region was around 71.5%.

Identification and cloning of the *snaC* gene. Degenerate oligonucleotide probes C1, C2, and C3 were designed from the N-terminal and two internal peptide sequences of the purified FMN reductase. None of them hybridized with the five previously described cosmids isolated with probes specific for PII$_A$ synthase genes. Hybridization of the library with C1, C2, and C3 probes allowed the identification of two cosmids which contained a common 4-kb *Bam*HI fragment hybridizing with the three probes. One cosmid, pIBV4, containing a 41-kb insert (Fig. 2), was further studied. The 4-kb *Bam*HI fragment from this cosmid was subcloned in pUC19 to give pVRC509 (Fig. 2). The nucleotide sequence of the 770-bp fragment internal to the 4-kb *Bam*HI fragment was determined (Fig. 3). Two adjacent ORFs showing a typical *Streptomyces* codon usage were found (Fig. 3). The average G+C content of the region was 76%. ORF2 started with a GTG at nucleotide 212, finished with TGA at nucleotide 731, and had a putative ribosome-binding site (AGGAG) 5 bp upstream of the start codon. ORF2 encoded a polypeptide of 176 amino acids with an M_r of 18,300. Only the 3′ end of ORF1 was present on the sequenced fragment.

The N-terminal sequence of the ORF2 product was identical to the N-terminal sequence of the purified FMN reductase (57), except that the N-terminal methionine was missing.



(A)

BamHI — Start ORF1 → — S/D — EcoRI — End ORF1 — End ORF2 — Start ORF2 — EcoRI — Start ORF3 → — S/D — Stop ORF3

ggatcctggcgtccgccgtcaagaactgaaccgaggagacacccacc ATG ACC GCA CCC CGC CGG GCC ATC ACC CTC GCC GGC ATC ATC GAC GGC CCC GGC GGC CAT GTG GCC GCC TGG CGC CAC CCG GCG ACC AAG GCG GAC GCC CAG CTC GAC TTC GAA TTC CAC CGC GAC AAC GCC CGC ACC CTC GAA CGC GGC CTG TTC GAC GCC GTG TTC ATC GCG GAC ATC GTC GCC GTG TGG GGC ACC CGC CTG GAC TCC CTG TGC CGC ACC TCG CGC ACC GAG CAC TTC GAA CCG CTC ACC CTG CTC GCC GCC TAC GCC GCG GTC ACC GAG CAC ATC GGC CTG TGC GCC ACC GCC ACC ACC ACG TAC AAC GAA CCG GCG CAC ATC GCC GCC CGC TTC GCC TCC CTC GAC CAC CTC AGC GGC GGC CGG GCC GGC TGG AAC GTC GTC ACC TCC GCC GCA CCG TGG GAG TCC GCC AAC TTC GGC TTC CCC GAG CAC CTG GAG CAC GGC AAA CGC TAC GAG CGG GCC GAG GAG TTC ATC GAC GTC GTC AAA AAA CTG TGG GAC AGC GAC GGC CGC CCC GTC GAC CAC CGC GGC ACC CAC TTC GAG GCC CCC GGC CCG CTC GGG ATC GCC CGC CCC CCG CAG GGC CGC CCC GTC ATC ATC CAG GCC GGC TCC TCG CCG GTG GGA CGC GAG TTC GCC GCC CGG CAC GCC GAG GTC ATC TTC ACC CGG CAC AAC CGG CTC TCC GAC GCC CAG GAC TTC TAC GGC GAC CTC AAC GCA CGC GTC GCC CGG CAC GGC CGC GAC CCC GAG AAG GTC CTC GTG TGG CCG ACC CTC GCG CCG ATC GTC GCC GCC ACC GAC ACC GAG GCG AAG CAG CGC CTG CAG GAA CTG CAG GAC CTC ACC CAC GAC CAT GTC GCC CTG CGC ACC CTT CAG GAC CAC CTC GGC GAC GTC GAC CTG AGC GCG TAC CCG ATC GAC GGG CCC GTC CCC GAC ATC CCG TAC ACC AAC CAG TCC CAG TCG ACG ACC GAG CGG CTG ATC GGC CTG GCC AGG CGC GAG AAC CTC AGC ATC CGC GAG CTG GCC CTG CGG CTG ATG GGC GAC ATC GTC GTC GGC ACA CCG GAG CAG CTC GCC GAC CAC ATG GAG AGC TGG TTC ACC GGC CGC GGC GCC GAC GGC TTC AAC ATC GAC TTC CCG TAC CTG CCG GGC TCC GCC GAC GAC TTC GTC GAC CAC GTG GTG CCC GAA CTG CAG CGC CGC GGC CTG TAC CGC TCG GGC TAC GAG GGC ACC ACC CTG CGG GCC AAC CTC GGC ATC GAC GCC CCC CGG AAG GCA GGT GCA GCG GCT tgacttccgtcctaAAG GCG GGG GAT TCC AGC GGT CGC CCG CTG GGG TTC CTG CTT CAC CGA CGA CCG CCC CGT CCG GGA GGA CTC CCG TTG AGG TCT TAT ACC GTC TCC ACA GGC CGA CGC CGC CAG CCC GGC GGC CAG GAT GTT GCG TGC CGC ATT CAC GTC GCG GTC ATG CAC AGC GCC GCA GTC GCA CGT CCA CTC CCG GAC GTT CAG CGG CAG CTT CCC GCG GAC CGT GCC GCA GGT TCC GCA CAG CTT GGA GCT GGG GAA CCA GCG GTC GAT CAC GAC GAG TTC GCG CCC ATA CCA GGC GCA CTT GTA CTC CAG CAT GGA GCG CAG TTC CGT CCA GGC CGC GTC GGA GAC GGC GGG CGC GAG CTT GCC GTT CTT CAG CAG GTT GCG GAC GGT GAG GTC CTC GAT CAC GAC CGT TTG GTT CTC ACG GAC GAG TCG AGT CGA CAG CTT GTG GAG GAA GTC GCA GCG CCG GTC GGT GAT

R V L R T S L K H L F D C R R D T : 247

CCG GGC GTG GAC GCG GGC GAC CTT GCG GCG GGC TTT CTT CCG GTT CGC CCA CCC 1849
R A H V R A V K R R A K K R N A S G 229

CTT CGC CTT GCG CGA CAC GTC CCG CTG AGC CTT CGC GAG GCG GGC GCG GTC ACG 1903
K A K R S V D R Q A K A L R A R D R 211

GCG CTC GTG CTT GGG GTT GGT GAT CTT CTC CCC GGT GGA CAG GGT CAC CAG GGA 1957
R E H K P N T I K E G T S L T V L S 193

GGT GAT CCC GGC GTC GAT GCC GAC GGC CGC CGT GGT GGC GGG CGC GGG GGT GAT 2011
T I G A D I G V A A T T A P A P T I 175

GGT GTC CTC GCA CAG CAG GGA CAC GAA CCA GCG GCC CGC ACG GTC GCG GGA CAC 2065
T D E C L L S V F W R G A R D R S V 157

GGT CAC CGT CGT CGG CTC CGC CCC TTC GGG AAG GGG ACG GGA CCA GCG GAT GTC 2119
T V T T P E A G E P L F R S W R I D 139

CAG GGG CTC CGC GGT CTT CGC CAG CGT GAG CTG TCC GTT ACG CCA CGT GAA GGC 2173
L P E A T K A L T L Q G N R W T F A 121

GCT GCG GGT GTA CTC GGC CGA CGC CCT GGA CTT TTT CCG CGA CTT GTA CCG CGG 2227
S R T Y E A S A R S K K R S K Y R P 103

GTA CTT CGA CCG CTT GGC GAA GAA GTT GGC GAA CGC CGT CTG CAA GTG CCG CAG 2281
Y K S R K A F F N A F A T Q L H R L 85

CGC CTG CTG GAG CGG GAC GGA GGA CAC CTC CGA GAG GAA GGC GAG TTC TTC GGT 2335
A Q Q L P V S S V E S L F A L E E T 67

CTT CTT CCA CTC CGT CAG CGC GGC GGA CGA CTG CAC GTA GGA GAC CCG GCG CTG 2389
K K W E T L A A S S Q V Y S V R R Q 49

CTC GCC GTA CCA GGC TCG CGT GCG CCC CTC AAG CGC CTT GTT GTA CAC GAG GCG 2443
E G Y W A R T R G E L A K N Y V L R 31

GAC ACA GCC GAA CGT GCG GGA CAG CTC AGC CGC CTG CTC GTC CGT GGG ATA AAA 2497
V C G F T R S L E A A Q E D T P Y F 13

← Start ORF2
GCG GTA CTT GAA AGC CCG CTT GAC CTG CTG CAT CAC gcctcacacgccatcagttcccg 2557
R Y K F A R K V C Q M N S/D 1

tgtgagcggcgggtgtctgccggtggttgcagacgccgaaccgcccttggcggcgattcgcccatccctgcc 2629

EcoRI — Start ORF3 →
ctgctccgcaagagcttcgtctcctccccggtctgaaggccggggtatccacgaaqggaattctg ATG ACC 2697
S/D M T 2

GCG CCC ATC CTC GTC GCC ACC CTC GAC ACC CGC GGC CCC GCC GCC ACC CTC GGC 2751
A P I L V A T L D T R G P A A T L G 20

ACG ATC ACC CGC GCC GTG CGG GCC GCG GAG GCC GCC GGA TTC GAC GCC GTC CTG 2805
T I T R A V R A A E A A G F D A V L 38

ATC GAC GAC CGG GCC GCC GCC GGC GTC CAG GGC CGG TTC GAG ACG ACG ACG CTG 2859
I D D R A A A A G V Q G R F E T T T L 56

ACC GCC GCG CTG GCC GCC GTC ACC GAG CAC ATC GGC CTG ATC ACC GCC CCG CTC 2913
T A A L A A V T E H I G L I T A P L 74

CCG GCC GAC CAG GCC CCC TAC CAC GTG TCC CGG ATC ACC GCC TCG CTC GAC CAC 2967
P A D Q A P Y H V S R I T A S L D H 92

CTC GCC CAC GGC CGC ACC GGC TGG CTC GCG AGC ACG GAC ACC ACC GAC CCC GAG 3021
L A H G R T G W L A S T D T T D P E 110

GGC CGC ACC GGC GAA CTC ATC GAC GTC GTC CGC GGC CTG TGG GAC AGC TTC GAC 3075
G R T G E L I D V V R G L W D S F D 128

GAC GAC GCC TTC GTC CAC GAC CGC GCC GAC GGC CTG TAC TGG CGG CTG CCC GCC 3129
D D A F V H D R A D G L Y W R L P A 146

GTC CAC CAA CTC GAC CAC CAG GGC AGG CAC TTC GAC GTG GCC GGC CCC CTC AAC 3183
V H Q L D H Q G R H F D V A G P L N 164

GTC GCC CGC CCG CCG CAG GGC CAC CCC GTC GTC GGC GTC ACC GGC CCC GCC CTC 3237
V A R P P Q G H P V V A V T G P A L 182

GCC GCG GCC GCC GAC CTC GTC CTG CTC GAC GAG GCG GCC GAC GCC GCC TCG GTG 3291
A A A A D L V L L D E A A D A A S V 200

AAG CAG CAG GCA CCG CAC GCC AAG ATC CTC CTG CCG CTG CCC GGC CCG GCC GCC 3345
K Q Q A P H A K I L L P L P G P A A 218

GAA CTG CCC GCC GAC AGC CCC GCG GAC GGC TTC ACG GTG GCG CTC ACC GGC TCC 3399
E L P A D S P A D G F T V A L T G S 236

GAC GAC CCG GTC CTG GCC GCG CTC GCC GCC CGG CCC GGC CGC CCG GAC CGC ACC 3453
D D P V L A A L A A R P G R P D R T 254

GCG GCC ACC ACC CTG CGC GAA CGC CTG GGC CTG GCC CGC CCC GAG AGC CGC CAC 3507
A A T T L R E R L G L A R P E S R H 272

Stop ORF3
GCC CTC ACC ACC GCC tgacgacccgtccgcccgctgcttcctggagagtcatgtcccgtcgcctgt 3573
A L T T A * 277

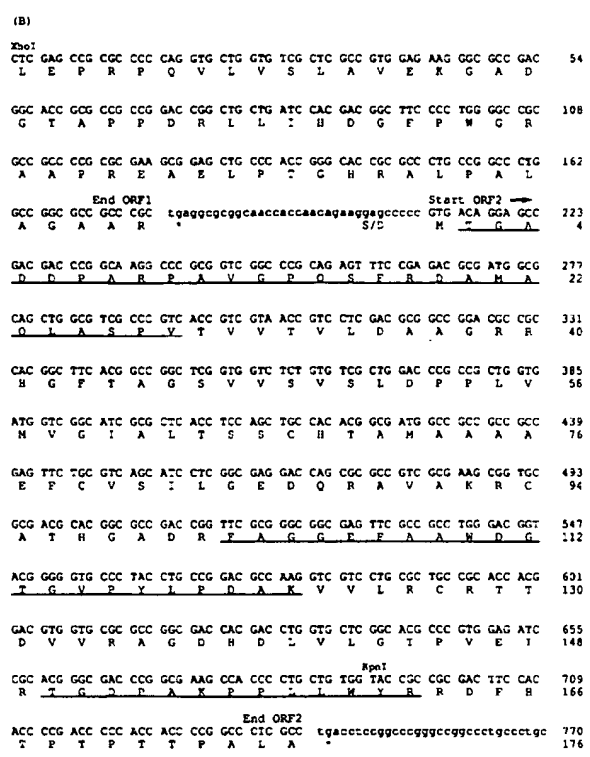


FIG. 3. (A) Nucleotide and derived amino acid sequences of a 3,573-bp region from the *Bam*HI-*Sst*I fragment carrying *snaA* and *snaB*. (B) Nucleotide sequence of a 770-bp fragment containing *snaC*. All the ORFs except ORF2 in panel A are on the strand shown. For ORF2, the amino acid sequence of the putative encoded protein was deduced from the other strand. The amino acid sequences determined from the N-terminal sequences and internal sequences of the purified SnaA, SnaB, and SnaC proteins are underlined. Noncoding DNA is represented in lowercase letters. The putative ribosome-binding sites (Shine-Dalgarno sequences [S/D] are shown. Relevant restriction sites are indicated over the nucleotide sequence.

Moreover, the two internal sequences of the protein matched exactly with internal segments of the ORF2 product (Fig. 3). The calculated molecular mass of ORF2 was smaller than the estimated 30 kDa of the purified FMN reductase. The identity of the deduced amino acid sequence with the three identified peptide sequences from the purified FMN reductase proves that ORF2 is the structural gene *snaC* encoding FMN reductase.

Sequence homology studies. No significant identity was found between SnaA, SnaB, and proteins in databases. However, the two proteins showed 37% identity over the whole sequences (Fig. 4). Gaps were introduced in the SnaB protein because of the smaller size of this subunit. High conserved regions between these gaps justified their presence.

The deduced protein corresponding to ORF401 was 50% identical with the product of ORF425 from IS*1136* from *S. erythraea*, the erythromycin producer (14). In addition, the entire gene products of *vsdF* from *Salmonella dublin* (35) and ORFE from *Salmonella typhimurium* (21), were 33% identical with the 100 N-terminal amino acids of the ORF401-encoded protein. The central 200 amino acids of ORF401 were 36% identical with the C-terminal portion of the gene product of 402 amino acids of an ORF found in the insertion sequence IS*891* from the cyanobacterium *Anabaena* sp. strain M131 (4).

Comparison of the *snaC* gene product with databases showed 39% identity with the *actVB* gene product (Fig. 4), involved in actinorhodin synthesis in *S. coelicolor* A(3)2 (17).

Disruption of the *snaA* gene in *S. pristinaespiralis*. To confirm the function of the SnaA protein, we disrupted the *snaA* gene in *S. pristinaespiralis* SP92 by single homologous recombination. *S. pristinaespiralis* protoplasts were transformed with 1 μg of pVRC505, containing an internal fragment of the *snaA* gene, as described in Materials and Methods. A few clones resistant to nosiheptide were studied. Southern blot analysis with pVRC505 as the probe showed that one clone named SP92::pVRC505 had stably integrated pVRC505 through homologous recombination (data not shown). This strain and SP92 (as control) were grown in fermentation broth and PI and PII components were extracted as described elsewhere (57). The mutant strain SP92::pVRC505 produced only PII$_B$, whereas the parental strain produced 80% PII$_A$ and 20% PII$_B$. PI production was identical in both strains. Western blotting showed that SnaA protein was absent from the mutant and, surprisingly, that SnaB was also undetectable (Fig. 5).

Homologous expression of *snaA* and *snaB* genes in *S. pristinaespiralis* SP120. SP120, isolated by chemical mutagenesis, had the same phenotype as that of SP92::pVRC505 for PII production, namely, accumulation of PII$_B$ and no immunologically cross-reacting bands with polyclonal antibodies raised against SnaA and SnaB proteins. Morever, SP120 did not produce PI. This mutant was used to perform complementation experiments with the *snaA* and *snaB* genes. Mutant SP120 was transformed with pVRC507, and nosiheptide-resistant clones were selected. Two transformants, SP120(pVRC507)-1 and SP120(pVRC507)-2, were studied further, with SP120 containing pIJ903 as a control. These clones regained the ability to oxidize PII$_B$ to PII$_A$, but complementation was partial, since PII$_A$ represented only 14% of the total PII in comparison to 80% in SP92. Expression of the *snaA* and *snaB* genes was confirmed by assay of PII$_A$ synthase activity (Table 2). PII$_A$ synthase activity of SP119 was assayed as the reference activity. FMN reductase activity was assayed as a control of the enzymatic assay. The results showed an increase in PII$_A$ synthase activity in SP120(pVRC507) clones; however and as predicted by the partial complementation, the increase was below the wild-type level (Table 2).

Heterologous complementation of the *S. coelicolor* B135 mutant by *snaC*. In order to demonstrate identity of the enzymatic activities of the SnaC protein and the product of the *actVB* gene, we expressed the *snaC* gene under the control of the *ermE** promoter in *S. coelicolor* B135, an *actVB* mutant. After transformation of the mutant B135 with unmethylated pVRC520, many transformants resistant to nosiheptide were isolated. These transformants were grown on R2YE medium (28), with nosiheptide as the selecting marker, and after 5 days, they became blue (data not shown). This color, specific for actinorhodin production (43, 53), did not appear when B135 was transformed with pIJ903.

DISCUSSION

The structural genes *snaA*, *snaB*, and *snaC* coding for the two enzymes involved in the last step of PII$_A$ biosynthesis were cloned, sequenced, and characterized. Three lines of evidence confirmed that *snaA* and *snaB* were the structural genes for PII$_A$ synthase: (i) disruption of *snaA* in *S. pristinaespiralis* resulted in strains producing only PII$_B$ and defective in SnaA and SnaB proteins; (ii) the SnaA and SnaB proteins were absent also in a PII$_A$ synthesis-deficient mutant SP120; and (iii) mutant SP120 was partially complemented for PII$_A$ production and PII$_A$ synthase activity by extra copies of *snaA* and *snaB*

FIG. 4. Alignments of amino acid sequences by the program of Kanehisa (31). (A) *S. pristinaespiralis* SnaA and SnaB proteins. (B) *S. pristinaespiralis* (SP) SnaC protein and *S. coelicolor* (SC) A(3)2 *actVB* gene product. Identical amino acids among the different sequences are shaded. Gaps in the alignments are indicated (-).

cloned in pIJ903. Although complementation of SP120 by the *snaA* and *snaB* genes was incomplete, this is unlikely to reflect the presence of a second mutation in SP120, because Sezonov (52) achieved complete complementation of this mutant with the *snaA* and *snaB* genes under the control of *ermE** promoter, using an integrative vector. The low level of complementation could be explained by the absence of a promoter in the cloned fragment and the transcription of *snaA* and *snaB* from the *tet* promoter of pIJ903.

Disruption of *snaA* led to the absence of both SnaA and SnaB proteins in Western blots. One hypothesis could be that the presence of SnaA stabilizes SnaB. However, because of the dramatic effect, it is more likely that *snaA* and *snaB* are cotranscribed. The transcript would then also include the antisense sequence of ORF401. This organization is similar to that for *eryAI* and *eryAII*, which encode multifunctional polypeptides involved in erythromycin biosynthesis in *S. erythraea* (14). These two genes are separated by an ORF, ORF425, similar to that of IS*891* from *Anabaena* sp. strain M131 in the opposite orientation. The ORF401 product is 50% identical to that of ORF425. In both cases, the low G+C content observed in the intergenic regions suggested an insertion of an external DNA fragment (14). Meanwhile, transcription of *snaA* and *snaB* in the *snaA* disruption mutant and the wild-type strain of *S. pristinaespiralis* should be examined to confirm this organization.

The conversion of PII_B to PII_A is similar to the reaction involved in the production of light by the luciferase of bioluminescent bacteria (for reviews, see references 46 and 47): luciferase catalyzing also the oxidation of a substrate (a long-chain aldehyde), coupled to the oxidation of a reduced flavin. The reaction also needs an NAD(P)H:FMN oxidoreductase. The luciferase is a heterodimer composed of two subunits, α with an M_r of 40,000 to 45,000 and β with an M_r of 35,000 to 40,000. We compared the small and large subunits of the PII_A synthase with the α and β subunits of luciferases from different bioluminescent bacteria, such as *Vibrio harveyi* (16) and *Vibrio fischeri* (18), and found only a weak identity between them. The highest scores obtained (17 to 19% identity) were always between SnaB and the α or β luciferase subunits, in the N-terminal regions. However, in all cases, a common motif was conserved (L-D-Q/H-M/L-S/A-X-G-R) in the N-terminal regions of these different proteins. Up to now, no role has been assigned to it. These proteins have similar functions, different substrates, and low identity. Nevertheless, an interesting point was the homology observed between SnaA and SnaB proteins. The same type of identity was observed between the α and β

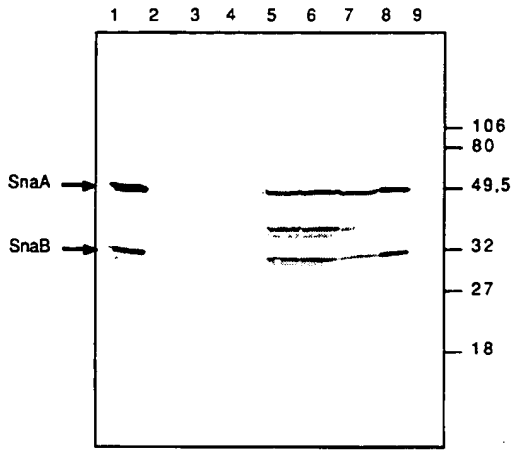


FIG. 5. Analysis of protein extracts after disruption of *snaA* gene in *S. pristinaespiralis* SP92 producing strain. Fermentation experiments were performed with SP92 and SP92::pVRC505 for 18, 20, and 22 h. Extracts were obtained by sonication of samples at each stage, and proteins were separated by electrophoresis with a SDS–12% polyacrylamide gel. The Western blot was obtained by using antibodies raised against the two subunits of the PII$_A$ synthase and stained with anti-rabbit immunoglobulin G-alkaline phosphatase conjugate. Lanes: 1 and 8, purified PII$_A$ synthase; 2, 3, and 4, extracts from 22, 20, and 18 h of fermentation of the mutant strain SP92::pVRC505, respectively; 5, 6, and 7, extracts from 22, 20, and 18 h of fermentation of the parental strain SP92, respectively; 9, molecular weight markers (in thousands). The positions of SnaA and SnaB are indicated to the left of the gel.

subunits of the different luciferases, which commonly shared 30% identity (29, 46, 47). However, the subunits of the luciferases are closer in size than are SnaA and SnaB. These observations suggested that both protein complexes shared a similar evolutionary pathway, probably a gene duplication event (46).

Restriction analysis of pIBV1, pIBV3, and pIBV4 indicated that *snaC*, the structural gene for FMN reductase, was at least 24 kb distant from the PII$_A$ synthase genes. This was surprising because of the involvement of the three genes in the same biosynthetic step and the fact that PII$_A$ synthase and FMN reductase were expressed at the same time during fermentation (57). However, pulsed-field electrophoresis analysis of the *S. pristinaespiralis* genome showed that the three genes were present on a common 500-kb *Ase*I fragment (3). Further studies will clarify if they are part of the same cluster.

The *snaC* gene encodes a protein of 173 amino acids. SnaC is strikingly similar to the product of the *actVB* gene of the actinorhodin cluster from *S. coelicolor*. Actinorhodin biosynthesis has been well studied (5, 11, 62). From the observation of Cole et al. (11), it was proposed that the *actVB* product was involved in a late step of the pathway, corresponding to the dimerization of an intermediate, likely to be dehydrokalafungin. Recently Kendrew et al. (34) have purified the corresponding enzyme and shown that it is a flavin: NADH oxidoreductase. Dimerization of kalafungin is proposed to be a phenolic oxidation (45) and probably involves an hydroxylation step identical to the reaction involved in PII$_B$-to-PII$_A$ conversion, requiring reduced FMN. Heterologous complementation of the *actVB* mutant, B135, by *snaC* confirms the recent results of Kendrew et al. (34), showing that the *actVB* product is also an FMN reductase. The calculated and estimated (57) M_rs of SnaC, 18,000 and 30,000, respectively, are the same as those observed for the *actVB* product, which has been shown to be a dimer (34). Thibaut et al. (57) were able to oxidize PII$_B$ to PII$_A$ with purified PII$_A$ synthase and the FMN reductase from *Photobacterium fischeri*, a bioluminescent bacteria (commercial preparation from Boehringer Mannheim). Luminous bacteria usually contain several flavin reductases (15, 30, 59), and recently, genes encoding major and minor NAD(P)H-flavin oxidoreductases involved in bioluminescence reactions from different bacteria were cloned and sequenced (37, 60, 61). Although these reductases were all associated with the emission of light, they could be divided in three groups displaying no significant homology (37, 60, 61). These results underlined the diversity of flavin reductases that could be involved in the same type of reaction. Comparison of SnaC with these different FMN reductases and with the major flavin reductase of *E. coli*, Fre (1, 54), showed no significant homology. Amino acid similarity observed between SnaC and the *actVB* product and analysis of their biochemical properties (34, 57) suggested that these two enzymes belong to the same FMN reductase family and are different from the different types of FMN reductases purified from bioluminescent bacteria and from the major flavin reductase of *E. coli*.

TABLE 2. PII$_A$ synthase and FMN reductase activities of *S. pristinaespiralis* strains

Strain	Activity (μmol/h/mg) of FMN reductase	Activity (nmol/h/mg) of PII$_A$ synthase
SP119	0.23	90
SP120(pIJ903)	0.17	<0.2
SP120(pVRC507)-1	0.16	3.3
SP120(pVRC507)-2	0.09	3.9

Thus, genes corresponding to the two-enzyme system catalyzing the last step of PII$_A$ biosynthesis have been cloned and characterized. Disruption or overexpression of these genes will allow us to construct strains that selectively produce each of the two main forms of PII, PII$_B$ and PII$_A$, respectively. Morever, because of the general clustering of genes involved in the same biosynthetic pathway in *Streptomyces* sp. (27), these results give us the possibility to identify other genes involved in pristinamycin biosynthesis by chromosome walking.

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QUINUPRISTIN/DALFOPRISTIN (RP 59500): A NEW STREPTOGRAMIN ANTIBIOTIC

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OBJECTIVE: To review the current knowledge on RP 59500 (quinupristin/dalfopristin, Synercid), a new streptogramin antibiotic, with respect to its pharmacology, pharmacokinetics, pharmacodynamics, mechanism of resistance, and in vitro inhibitory and bactericidal activity.

DATA SOURCES: A MEDLINE search using the keywords RP 59500, pristinamycin, virginiamycin, and streptogramin was performed. Relevant abstracts presented at recent scientific conferences also were consulted.

STUDY SELECTION: Because RP 59500 is a relatively new investigational agent, relevant in vitro and animal studies were selected. All available human studies were included as well.

DATA EXTRACTION: Data from in vitro and in vivo studies were included, with particular emphasis on human studies.

DATA SYNTHESIS: RP 59500 is a new injectable streptogramin antibiotic consisting of a mixture of 2 synergistic pristinamycin compounds. RP 59500 possesses in vitro inhibitory and bactericidal activity against most isolates of gram-positive organisms including vancomycin-resistant *Enterococcus faecium*, selected gram-negative bacteria, and most anaerobic organisms. Based on preliminary data, the drug appears to be metabolized rapidly and extensively while exhibiting a significant postantibiotic effect. Data from ongoing clinical trials suggest that RP 59500 is well-tolerated except for mild injection site irritations. However, before the role of RP 59500 within the vast armamentarium of antimicrobials can be elucidated, additional studies need to be conducted to document its clinical efficacy.

CONCLUSIONS: Based on in vitro susceptibility testing, in vivo studies, and preliminary clinical data, RP 59500 may be an alternative to the glycopeptides, especially for inherently resistant organisms. Further studies are needed to confirm this agent's in vitro activity and to establish its clinical efficacy.

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QUINUPRISTIN/DALFOPRISTIN (RP 59500) is a novel investigational antimicrobial belonging to the family of streptogramin antibiotics, which are naturally occurring compounds isolated from *Streptomyces pristinaspiralis*.[1] The streptogramin family comprises several series of similar antibiotics such as the mikamycins, the pristinamycins, the oestreomycins, and the virginiamycins.[2] Oral pristinamycin (Pyostacine) is a streptogramin antibiotic that has been used in Europe for many years, primarily in the management of staphylococcal infections.[1] In this article, the pharmacologic and bacteriologic properties of RP 59500 are discussed, along with its current status in clinical trials and potential therapeutic applications.

Chemistry

The streptogramin family of antibiotics can be divided into 2 different groups. Group A (or M) are polyunsaturated cyclic peptidolide compounds including pristinamycin IIA, while group B (or S) compounds are cyclic hexadepsipeptides that include pristinamycin IA.[2] RP 59500 consists of a combination of quinupristin (RP 57669) and dalfopristin (RP 54476) in its naturally occurring ratio of 30:70 (w/w). Quinupristin and dalfopristin, whose chemical structures are shown in Figure 1, are derivatives of pristinamycin IA and IIA, respectively. Molecular modifications of the natural compounds were required to increase their aqueous solubility, thus enabling the drug to be formulated as an injectable for use in the management of serious infections.[1]

Individual pristinamycin compounds exhibit bacteriostatic activity against gram-positive bacteria.[3] However, combinations using a compound from each group of the streptogramin family usually result in synergistic and bactericidal activities. This has been demonstrated with the quinupristin/dalfopristin combination. In vitro studies have documented lower minimum inhibitory concentrations (MIC) against isolates of staphylococci and streptococci for quinupristin/dalfopristin than with either component alone.[4,5] For example, the MIC for 90% of the strains tested (MIC_{90}) against methicillin-resistant *Staphylococcus aureus* (MRSA) for RP 57669, RP 54476, and RP 59500 were 8, more than 16, and 1 mg/L, respectively.[5] In addition, the fractional inhibitory concentration (FIC) indices to various ratios of RP 54476 and RP 57669 against *S. aureus* of several resistance phenotypes were consistently less than 0.5.[6] The FIC index is a calculated value that reflects the inhibitory activity of a certain combination of antimicrobial agents compared with each of the individual agents alone. An FIC index of no more than 0.5 indicates that the 2 tested drugs are synergistic. This synergy associated with RP 59500 has been proposed to be caused by the

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Quinupristin/dalfopristin (Synercid, Rhône-Poulenc Rorer).

drug's mechanism of action, which appears to be similar to that of the aminoglycosides, and is thought to be a result of sequential binding of dalfopristin and quinupristin to different sites on the 50S subunit of bacterial ribosomes.[3] The proposed mechanism of action is that the binding of dalfopristin alters the conformation of the ribosome such that its affinity for quinupristin is increased. This results in a stable ternary drug–ribosome complex, and the newly synthesized peptide chains cannot be extruded from the ribosome of that complex. Consequently, protein synthesis is interrupted, thus leading to cell death.

Spectrum of Antibacterial Activity

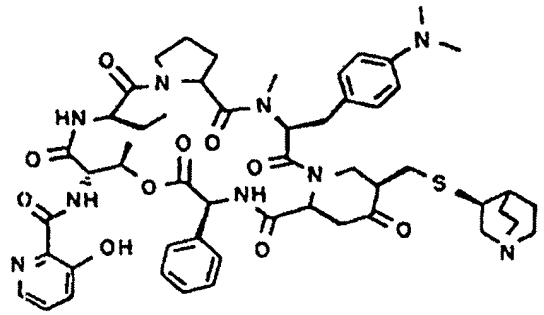
Numerous in vitro susceptibility studies have been performed with RP 59500.[4,5,7-17] In general, RP 59500 has been reported to possess inhibitory activity against *S. aureus* (including methicillin-resistant strains), coagulase-negative staphylococci, streptococci (including penicillin-resistant *Streptococcus pneumoniae*), enterococci, *Neisseria* spp. *Haemophilus influenzae, Moraxella catarrhalis, Legionella* spp., and *Listeria monocytogenes*. RP 59500 is also active against most gram-positive as well as gram-negative anaerobic organisms from several genera including *Bacteroides, Prevotella, Fusobacterium, Clostridia, Actinomyces, Peptostreptococcus*, and *Lactobacilli*. The results of the various in vitro susceptibility studies are summarized in Table 1.[4,5,7-14,16,17] Because RP 59500 primarily possesses activity against gram-positive organisms, comparative MICs to vancomycin and macrolide antibiotics are presented in Tables 2 and 3.[4,5,7,9,12-14,16,17] Relatively speaking, RP 59500 and vancomycin have similar and consistent MICs against *S. aureus*, streptococci (e.g., *S. pneumoniae*), as well as their average achievable serum concentrations to MIC ratio, with RP 59500 having somewhat lower MICs for *S. epidermidis* than does vancomycin. Both vancomycin and RP 59500 have MICs against *Enterococcus faecalis* and *Enterococcus faecium* that are within the range of achievable serum concentrations. However, as indicated in Table 2, vancomycin-resistant strains of enterococci, especially *E. faecium*, still appear to be inhibited by achievable serum concentrations of RP 59500. When compared with erythromycin and other macrolides (e.g., azithromycin, clarithromycin), most strains of staphylococci and enterococci that were reported to be resistant to erythromycin remained sensitive to RP 59500 (Tables 2 and 3). Against streptococci, using *S. pneumoniae* as an example, RP 59500 and erythromycin appear to have similar inhibitory activity. However, RP 59500 was also active against erythromycin-resistant strains of *S. pneumoniae*.[4,8,12] A similar trend of relative inhibitory activity is seen when RP 59500 is compared with azithromycin and clarithromycin. RP 59500 has no activity against members of the Enterobacteriaceae family or other gram-negative bacilli such as *Pseudomonas aeruginosa*.[12]

RP 59500 has been shown to antagonize bactericidal activities of oxacillin and gentamicin against the American Type Culture Collection (ATCC) 29213 strain of *S. aureus*.[7] Against *E. faecalis* (ATCC 29212 strain), the combination of RP 59500 and ampicillin demonstrated antagonism, but combination with gentamicin displayed indifference. When RP 59500 was combined with ciprofloxacin, cefotaxime, or gentamicin, the gram-negative activity of these agents was not altered.[5]

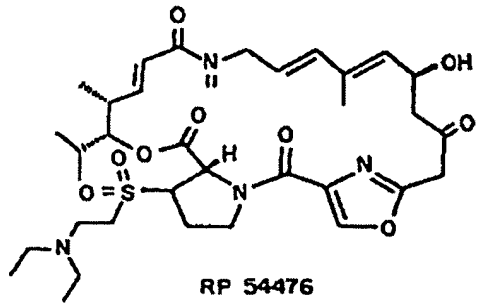
Table 1. Summary of In Vitro Activities of RP 59500

ORGANISM	MIC_{90}* (mg/L)	RANGE (mg/L)	REF.
Staphylococcus aureus			
methicillin-sensitive	0.62	<0.10–2.0	4,5,7,8,12,13,17
methicillin-resistant	0.87	0.03–4.0	4,5,7,8,12,13,17
Staphylococcus epidermidis			
methicillin-sensitive	0.41	0.03–4.0	5,7,12,17
methicillin-resistant	0.40	0.03–4.0	5,7,12,17
Streptococcus pyogenes	0.39	0.03–1.0	5,7,12,13
Streptococcus agalactiae	0.38	≤0.063–1.0	4,5,7,12
Viridans streptococci	1.3	0.25–4.0	5,7,12
Streptococcus pneumoniae	0.71	0.025–2.0	4,5,7,8,13
penicillin-resistant	1.0	<0.125–2	11
Enterococcus faecalis			
vancomycin-sensitive	7.1	0.25–32	4,5,7,9,12,13
vancomycin-resistant	32	4–32	9
Enterococcus faecium			
vancomycin-sensitive	2.8	0.25–8	4,7,9
vancomycin-resistant	4.7	0.06–32	9,14,16
Neisseria meningitidis	0.31	≤0.12–1.0	5,12
Neisseria gonorrhea			
beta-lactamase (+)	0.67	0.015–2.0	5,12
beta-lactamase (–)	1.0	0.015–2.0	12
Moraxella catarrhalis	1.0	≤0.12–1.0	5,12
Haemophilus influenzae			
beta-lactamase (+)	2.6	0.25–8.0	5,12
beta-lactamase (–)	4.0	2.0–4.0	12
Legionnella pneumophilia	0.34	0.008–1.0	10
Listeria monocytogenes	2.0	2.0–16.0	5
Bacteroides fragilis	4.0	2.0–4.0	5
Clostridium perfringens	0.25	≤0.012–0.25	5

MIC_{90} = minimum inhibitory concentration for 90% of the strains tested.
*When applicable, this represents a weighted mean MIC_{90} calculated from the various references, if a specific MIC_{90} value was reported.



RP 57669



RP 54476

Figure 1. Graphic structures of quinupristin (RP 57699) and dalfopristin (RP 54476). Reprinted with permission.[3]

Table 2. Comparative Inhibitory Activity of RP 59500, Vancomycin, and Erythromycin

ORGANISM	MIC_{90}* (range) (mg/L) ERYTHROMYCIN	VANCOMYCIN	RP 59500	REF.
Staphylococcus aureus				
methicillin-sensitive	1.3 (<0.05–>100)	1.0 (0.25–2.0)	0.62 (<0.1–2)	4,7,12,13,17
methicillin-resistant	>16 (0.1–>128)	1.3 (0.25–2.0)	0.9 (0.03–4.0)	
Staphylococcus epidermidis				
methicillin-sensitive	16 (0.03–≥64)	1.5 (0.25–2.0)	0.4 (0.03–4.0)	7,12,17
methicillin-resistant	>16 (0.06–≥64)	2.0 (0.25–2.0)	0.27 (0.03–4.0)	
Streptococcus pneumoniae	0.05 (<0.006–0.25)	0.5 (≤0.063–1)	0.68 (0.025–1.0)	4,7,12,13
erythromycin-resistant	>32 (4–>32)	0.5 (0.25–0.5)	1.0 (0.25–1.0)	12
Enterococcus faecalis				
vancomycin-sensitive	32 (≤0.063–>256)	2.6 (0.25–8.0)	7.0 (0.25–32)	4,7,9,12,13
vancomycin-resistant	>256 (2–>256)	>256 (16–>256)	32 (4–32)	9
Enterococcus faecium				
vancomycin-sensitive	>128 (2–>256)	3.0 (0.25–8)	2.8 (0.25–8)	4,7,9
vancomycin-resistant	>256 (2–>256)	>256 (16–>512)	4.7 (0.06–32)	9,14,16

MIC_{90} = minimum inhibitory concentration for 90% of the strains tested.
*When applicable, this represents a weighted mean MIC_{90} from the various references, if a specific MIC_{90} value was reported.

Resistance

The most commonly known resistance to streptogramins is termed the MLS_B (macrolide, lincosamide, and streptogramin group B) resistance conferred by the *erm* gene.[18,19] This gene encodes for an enzyme that dimethylates 1 adenine residue in the 23S rRNA, which results in decreased binding of macrolides (erythromycin), lincosamides (clindamycin), and streptogramin B (pristinamycin). However, because RP 59500 is a combination of streptogramin A (RP 54476) and streptogramin B (RP 57669), one would expect that RP 59500 would remain active against MLS_B-positive organisms. This was demonstrated by Leclercq et al.[20] with both constitutive and inducible strains of MLS_B-positive *S. aureus*. Other mechanisms of resistance to streptogramin involve inactivating enzymes such as streptogramin-A acetyltransferase and streptogramin B hydrolase.[18,19] Both of these enzymes inactivate either group A or B streptogramin, respectively, and were shown not to be problematic to RP 59500 unless both enzymes were present in the same strain of bacteria. The incidence of such RP 59500-resistant strains of staphylococci has been estimated to be less than 5% in France.[20] Thus, cross-resistance to macrolides may be seen, though as mentioned, this was probably a relatively rare and clinically insignificant occurrence. Furthermore, an ATP-binding protein also has been found in certain strains of staphylococci that actively pump (i.e., efflux) streptogramin and macrolide antibiotics out of the cell, constituting the final known mechanism of resistance to the streptogramin antibiotics.[19] Thus far, cross-resistance between the streptogramin antibiotics with other classes of drugs such as the glycopeptides, beta-lactams, and aminoglycosides have not been reported.

Pharmacokinetics

In 1992, the disposition of RP 59500 was studied in humans in a Phase I, double-blind, randomized, placebo-controlled study.[21] Twenty-six healthy men (mean age 28.8 ± 5.3 y) received 3 different doses of RP 59500 ranging from 1.4 to 29.4 mg/kg intravenously over 1 hour. The elimination half-lives ($t_{1/2}$) of RP 59500 and quinupristin, when RP 59500 was given in doses of 12.6–29.4 mg/kg, were 1.27–1.53 and 0.56–0.61 hours, respectively. Dalfopristin was found to be metabolized rapidly to RP 12536, a naturally occurring pristinamycin IIA; thus, its $t_{1/2}$ could not be evaluated. Maximum serum concentrations of RP 59500, which were evaluated by using the amount of total serum antimicrobial activity, ranged from 0.95 to 24.20 mg/L. The investigators also reported a positive correlation between the serum concentrations with the administered dose. However, correlation was not reported between the dose given and the area under the curve. RP 12536 was thought to be active because measurable antimicrobial activity of RP

Table 3. Comparative Minimum Inhibitory Concentrations of RP 59500 and the Macrolide Antibiotics

ORGANISM	MIC_{90}* (range) (mg/L) ERYTHROMYCIN	CLARITHROMYCIN	AZITHROMYCIN	RP 59500
Staphylococcus aureus				
erythromycin-sensitive	0.5 (0.12–4.0)	0.5 (0.06–4.0)	8.0 (0.25–>16)	1.0 (0.06–1.0)
erythromycin-resistant	>16 (>16)	>16 (4–>16)	>16 (>16)	1.0 (0.12–2.0)
methicillin-resistant	>16 (0.5–>16)	>16 (0.06–>16)	>16 (0.25–>16)	1.0 (0.06–2.0)
Staphylococcus epidermidis				
erythromycin-sensitive	1.0 (0.25–4.0)	0.25 (0.03–0.5)	4 (0.5–4.0)	0.5 (0.12–1.0)
erythromycin-resistant	>16 (>16)	>16 (2–>16)	>16 (>16)	0.5 (0.12–0.5)
methicillin-resistant	>16 (8–>16)	>16 (16–>16)	>16 (>16)	0.5 (0.12–1.0)
Streptococcus pneumoniae	0.25 (0.03–1.0)	0.12 (0.03–2.0)	0.25 (0.06–1.0)	1.0 (0.25–2.0)

MIC_{90} = minimum inhibitory concentration for 90% of the strains tested.
*All MIC_{90} values were obtained from reference 5.

59500 (an indirect method of determining the $t_{1/2}$ of RP 59500) persisted for up to 6 hours in a dose-dependent manner despite the absence of measurable quinupristin or dalfopristin in the serum using an HPLC method. The contribution of RP 59500's long postantibiotic effect (PAE) to this prolonged activity was not evaluated. Consequently, the authors pointed out the need for further studies regarding the relationship between total serum antimicrobial activity of the parent drug, measured concentrations of RP 59500, and the antimicrobial activity of the metabolite.

In a smaller study involving 6 healthy men, Gaillard et al.[22] determined that an average of 75% and 77% of radioactivity from the administered dose of quinupristin and dalfopristin, respectively, were recovered in the feces. Similarly, only 15% of the radioactivity from the dose of quinupristin and 19% of dalfopristin were excreted into the urine over the first 3 hours. These preliminary results, in addition to the fact that only small amounts of unchanged dalfopristin can be found in either urine or feces, would suggest that both dalfopristin and quinupristin are metabolized extensively. Two of the metabolites of RP 59500, RP 12536 and RP 100391, were shown to possess in vitro antimicrobial activity. Clinical significance of these active metabolites remains to be established. Finally, the authors reported that protein binding for quinupristin and dalfopristin was 23–32% and 50–56%, respectively. Additional pharmacokinetic studies are currently underway (personal communication, B Kreter PharmD, Associate Director, Anti-Infectives, Rhône-Poulenc Rorer, March 30, 1995.)

Pharmacodynamics

The antibacterial activity of RP 59500 does not seem to be influenced significantly by the size of inoculum used in susceptibility testing.[5,7] A change of inoculum size from 5×10^5 to 5×10^7 cfu/mL was shown to result in minimal (e.g., 1 dilution step), if any, changes in the MICs of the organisms.[5,7] However, increasing the pH of the incubating broth from 7 to 8 in vitro resulted in a 2–3 times increase of the MIC values for RP 59500 against various staphylococci.[5] The addition of human serum raised the MIC values by 2–4 dilution steps and the addition of albumin 5% to the incubating medium resulted in an increase of minimum bactericidal concentration (MBC) values 4–8-fold.[5,7] These alterations in MICs and MBCs secondary to modifications in the incubating medium were attributed to the change in unbound fraction of RP 59500 in the presence of albumin or human serum. Available protein binding data suggest that both quinupristin and dalfopristin are, at best, only moderately bound to plasma protein and may not have contributed significantly to this alteration.[22] In terms of bactericidal activity, MBC values of RP 59500 are usually 1–4 times the MIC values for most susceptible organisms. Larger differences were sometimes, though not always, seen with MRSA, methicillin-sensitive *S. aureus* (MSSA), and enterococci than with streptococci.[7,8,17] Thus, tolerance to quinupristin/dalfopristin does not seem to occur.

Against staphylococci and enterococci, RP 59500 demonstrated slow progressive killing kinetics over the first 8 hours in vitro, with a trend toward more rapid killing as drug concentration increases from 1 to 8 times the MIC of the tested strains.[8] Minimal additional bactericidal activity was seen beyond 8 hours unless high drug concentrations were used. With respect to streptococci, RP 59500 was found to be rapidly bactericidal.[13,17] Furthermore, prolonged PAE has been demonstrated with quinupristin/dalfopristin. Nougayrede et al.[23] showed a PAE of 2–3 hours with RP 59500 at a drug concentration equal to the MIC of 4 strains of *S. aureus* when exposed for as little as 15–30 minutes. When drug concentration was raised to 4 times the MIC and the exposure time to 80 minutes, a PAE of 7–8 hours was observed against the same 4 strains of *S. aureus*. For RP 59500, the ratio of the drug concentration to the MIC seems to have a larger effect on the duration of PAE than the exposure time of the organism to the antibiotic.[23] Consequently, the long duration of PAE and the potentially prolonged antibacterial activity resulting from the active metabolite(s) have led to the use of a twice- or thrice-daily dosing regimen in Phase II and Phase III studies.[21,24]

The cellular uptake of RP 59500 has been evaluated in an in vitro study. Desnottes and Diallo[25] determined that macrophage uptake of both quinupristin and dalfopristin was rapid and extensive. After 2 hours, intracellular concentrations of quinupristin and dalfopristin were determined to be 50 and 34 times that of extracellular drug concentrations, respectively. Uptake was found to be increased by increasing the pH of the medium, a fact that may have potential therapeutic importance in the use of RP 59500 to eradicate intracellular organisms (e.g., *S. aureus*) located within necrotic or abscessed tissues. Indeed, the authors reported that more than 70% of the staphylococci within macrophages were eradicated within 2 hours of exposure to RP 59500.

Adverse Drug Reactions

In the only human pharmacokinetic study published thus far with intravenous RP 59500, no cardiac, respiratory, hematologic, or biochemical abnormalities were reported in 26 healthy volunteers.[21] RP 59500 was, however, associated with mild-to-moderate local reactions such as itching, pain, and burning that was self-limited and not associated with measurable histamine release. These local reactions were reported during 19 of the 53 infusions administered. Headaches occurred in 7 of the subjects, with similar distribution between placebo- and RP 59500-treated patients. Diarrhea (3 subjects) and vomiting (2) were the only other adverse effects reported. Preliminary data from ongoing Phase II and Phase III studies also have reported increases in liver function test results, with only 1 patient thus far having an increase of more than 5 times the upper limit of normal.[24] This seems to be an infrequent occurrence and symptomatic liver dysfunction has not been documented in patients with increased liver function test results.

Potential Therapeutic Uses and Current Status

In a rabbit endocarditis model, RP 59500 was shown to significantly decrease ($p < 0.001$) colony counts in vegetations infected by certain strains of MRSA.[26] In 2 cases, the vegetations were reported to be sterile after RP 59500 treatment. The bactericidal activity of RP 59500 was found to be similar or somewhat less than that of vancomycin, depending on the strain of MRSA chosen. However, it was noted that the mean peak serum concentration of RP 59500 achieved from the study dose of 20 mg/kg was only 1.9 ± 0.8 mg/L. This concentration is much smaller than

the MBC values of the various strains of MRSA involved and thus may have explained the inconsistent results. In another in vitro study, an intravenous dose of RP 59500 was effective in sterilization of fibrin clots infected by MRSA/MSSA, but was only somewhat effective in decreasing the colony counts in clots infected with methicillin-sensitive or methicillin-resistant *S. epidermidis*, and was entirely ineffective in reducing colony counts in fibrin clots infected with *E. faecalis*.[27] These results could be explained by the low drug concentrations achieved within the clot and the known relative intrinsic resistance of *E. faecalis* to RP 59500. However, autoradiographic studies have demonstrated that RP 59500 is capable of penetrating even the core of infected vegetations in vivo.[28] In addition, Kang and Rybak[29] recently reported that using in vitro killing studies with MSSA (ATCC strain 25923) and a clinical MRSA isolate, the time required to decrease the colony counts by 99.9% was significantly shorter with RP 59500 than with vancomycin. RP 59500 also was more effective than vancomycin in reducing the number of colony-forming units in an infected fibrin clot model with the same strains of staphylococci. More interestingly, it was observed that the combination of vancomycin and RP 59500 was significantly more effective that either agent alone in decreasing colony-forming units with both the in vitro studies and the fibrin clot model. Synergism between vancomycin and RP 59500 was seen with the in vitro experiments, but not with the fibrin clot models. As the authors pointed out, the different mechanisms of action of both agents may result in synergistic activity. Of interest, the authors also reported the development of resistance with the 2 staphylococci strains toward RP 59500. The MICs of the mutated strains had risen from a baseline of 0.19 mg/L to as high as 6.25 mg/L, which were noted to be stable over time (i.e., MICs did not revert back to baseline values). The spontaneous mutations leading to the resistant strains were found to have occurred at very low frequencies (approximately 10^{-8} to 10^{-10} order of magnitude) and were associated with cross-resistance to erythromycin, but not to lincomycin. Furthermore, combination treatment with vancomycin and RP 59500 was found to prevent the development of this resistance. The clinical significance of this rare development of resistance remains unknown and requires clarification with further clinical studies.

Given these in vivo and in vitro results and the spectrum of activity of RP 59500, it would seem that the drug has a potential role in the management of MRSA, enterococci (particularly *E. faecium*), and perhaps methicillin-resistant *S. epidermidis* infections in patients who cannot receive conventional therapy such as vancomycin. Furthermore, the availability of an injectable formulation, rather than the oral pristinamycin marketed in Europe, would be advantageous in the management of serious infections and/or patients who cannot tolerate oral antibiotic administration.

Clinical trials (Phases II and III) are currently underway in which RP 59500 is being investigated for its use in the management of pneumonia, catheter-associated bacteremia, and skin and soft tissue infections.[25] The results of studies with 72 patients who have been treated with RP 59500 under an investigator-sponsored investigational new drug program have been reported.[30] RP 59500 was used in these patients for the management of life-threatening infections caused by vancomycin-resistant *E. faecium* isolated from various sites such as the abdomen, wounds, cerebral spinal fluid, pleural fluid, lower respiratory tract, blood, catheter, urinary tract, cardiac valves, and bone. Clinical and bacteriologic cures of 78% and 82%, respectively, were reported with appropriate documentation for 52 of these patients.

Summary

RP 59500 is a unique antibiotic of the streptogramin family that is active primarily against gram-positive organisms. RP 59500 also has activity against selected gram-negative and most anaerobic organisms. Given the array of efficacious agents currently available for the management of infections resulting from gram-negative and anaerobic organisms, it seems that the potential role for RP 59500 would be in the treatment of gram-positive infections, especially those resulting from resistant strains such as staphylococci and *E. faecium*. If additional human studies corroborate these in vitro activities of RP 59500, then the drug may be considered as a viable alternative in the management of infections caused by resistant organisms in patients who are refractory to or intolerant of conventional therapy. Until the results of such studies are available, the use of RP 59500 should be considered only as investigational. ≃

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EXTRACTO

OBJETIVO: Revisar la farmacología, farmacocinética, farmacodinamia, mecanismo de resistencia, y actividad inhibitoria y bactericida in vitro del RP 59500 (quinupristin/dalforpristin, Synercid), un nuevo antibiótico estreptogramino.

FUENTES DE INFORMACIÓN: Base de datos MEDLINE empleando las palabras claves RP 59500, pristinamicina, virginiamicina, y estreptogramino. También extractos presentados en conferencias científicas recientes.

SELECCIÓN DE FUENTES DE INFORMACIÓN: Puesto que el RP 59500 es un agente en investigación relativamente nuevo, se seleccionaron estudios in vitro, en animales y en humanos.

METODO DE EXTRACCIÓN DE INFORMACIÓN: Se incluyeron datos prevententes de estudios in vivo e in vitro, con particular énfasis en estudios en humanos.

SÍNTESIS: RP 59500 es un antibiótico estreptogramino nuevo que consiste de una mezola de 2 compuestos de la pristinamicina de acción sinergística. RP 59500 posee actividad inhibitoria y bactericida contra la mayoría de los organismos gram-positivos, incluyendo *Enterococcus faecium* resistente a la vancomicina, cierras bacterias gram-negativas, y la mayoría de los organismos anaeróbicos. Información preliminar parece demostrar que este medicamento es metabolizado rápida y extensivamente mientras que posee un efecto postantibiótico significativo. Información obtenida de estudios clínicos en curso sugieren que el RP 59500 es bien tolerado excepto por irritaciones leves reportadas en el sitio de inyección. Sin embargo, antes de que el papel del RP 59500 pueda ser elucidado dentro del extense armamento de agentes antimicrobianos, se requieren estudios adicionales que documenten su eficacia clínica.

CONCLUSIÓNES: Basándose en pruebas de susceptibilidad in vitro, estudios in vivo, y datos clínicos preliminares, RP 59500 puede ser una alternativa a los glicopéptidos, especialmente en el caso de organismos inherentemente resistentes. Se requieren estudios adicionales que confirmen su actividad in vitro y su eficacia clínica.

ENCARNACIÓN C SUÁREZ